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Genomic Characterization of Carbapenemase-Producing Enterobacteriaceae Using Whole Genome Sequencing Data

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Abstract: Background/Objectives: Multidrug-resistant (MDR) infections pose a significant public health threat by worsening patient outcomes, contributing to hospital outbreaks, and increasing the health and economic burden. Advanced genomic tools enhance the detection of resistance genes, virulence factors, and high-risk clones, thus improving the management of MDR infections. In the Autonomous Community of Aragon, the carbapenemase-producing Enterobacteriaceae (CPE) diversity and incidence have increased during the last years. This study analyses CPE trends at a tertiary hospital in Spain from 2021-2023, aiming to optimize personalised medicine. **Methods**: CPE isolates were the first isolate per patient, year, species, and carbapenemase from January 2021 to December 2023. Antibiotic susceptibility testing was performed by broth microdilution method. Whole genome sequencing (WGS) was performed using Illumina. The genomes were thoroughly analysed to determine their complete taxonomic classification, resistoma and plasmidome composition, and sequence typing using a suite of bioinformatics approaches. **Results**: Between 2021 and 2023, 0.4% of all isolates were CPE. The CPE rate tripled in 2022 and doubled again in 2023. The most common species was *Klebsiella pneumoniae* (51.8%) and the most common carbapenemase was bla_{oxa-48}. WGS revealed concordant species identification and detailed carbapenemase distribution. Resistance rates to critical antibiotics such as carbapenems were variable but in most cases above 70%. Genetic diversity was observed in WGS and phylogenetic analyses, with plasmids often mediating carbapenemase dissemination. **Conclusions**: The increasing rate of CPE in healthcare settings highlights a critical public health challenge, with limited treatment options. Genomic characterization is essential to understand resistance mechanisms, aid therapy, limit outbreaks and improve precision medicine.

Keywords: carbapenem-resistant enterobacteriaceae; whole genome sequencing; drug resistance; microbial; computational biology

1. Introduction

Infections caused by multidrug-resistant (MDR) microorganisms represent a significant global public health issue [1,2]. The isolation of MDR microorganisms from clinical or epidemiological samples often correlates with poorer prognoses and increased morbidity, particularly when other virulence factors are present [3]. Furthermore, many of these microorganisms have the ability to spread within communities and hospitals, leading to outbreaks and epidemics [4,5]. Accurate characterisation of resistance determinants, virulence factors, mobile genetic elements, and the clonal relationships among isolates can be achieved using high-throughput sequencing and bioinformatics tools. This enables the detection and tracking of high-risk clones, phenotypic correlation with

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antimicrobial susceptibility testing, and control of hospital outbreaks. These results complement those obtained by traditional methods and have proven useful in the management of infections and outbreaks caused by MDR microorganisms worldwide, leading to their increasingly wide spread use in clinical microbiology and public health increasingly widespread [6,7].

In 2017, the World Health Organization (WHO) published a priority list of pathogens to guide research and development of new antibiotics, categorising carbapenemase-producing Enterobacteriaceae (CPE) as critical priority [8]. CPE are characterized by causing difficult-to-treat infections, increased morbidity and mortality, and high transmissibility, thus necessitating their active surveillance. Additionally, their prevalence is high in certain countries, increasing the likelihood of transmission, particularly in hospital settings [9].

In 2021, as part of the National Plan against Antibiotic Resistance (PRAN) in Spain, the Network of Laboratories for the Surveillance of Resistant Microorganisms (RedLabRA) was created , with the primary objective of achieving comprehensive and high quality microbiological diagnostics [10]. This network integrates genomic sequencing in all cases of infection or colonisation by antibiotic-resistant microorganisms under surveillance in the National Health System (currently including *Escherichia coli, Klebsiella pneumoniae*, and *Enterobacter cloacae* complex producing carbapenemases).

In the Autonomous Community of Aragón, Spain, the isolation of CPE has been sporadic and was previously limited to OXA-48-producing *K. pneumoniae*, leading to hospital outbreaks that were identified and contained [11]. However, in recent years, both the number and diversity of bacterial species and carbapenemases have steadily increased, creating new paradigms for their surveillance, detection, treatment, and interdisciplinary communication. These findings may be related to the aftermath of the COVID-19 pandemic and the forced displacement caused by the war in Ukraine [12,13], which has altered our hospital's medical care and referral system of military and civilian. In this sense, the Miguel Servet University Hospital (HUMS) has deemed it essential to monitor CPE and vancomycin-resistant *Enterococcus* due to the increasing incidence and impact of their spread in the community.

The integration of genomic data analysis into clinical microbiology, with a translational perspective, could yield results that add significant value to the care of patients with MDR microbial infections and advance the pursuit of personalized medicine [14].

In the ever-evolving landscape of global healthcare, the rise of multidrug resistant bacteria has become a pressing concern, posing a significant threat to public health and challenging the efficacy of conventional antimicrobial treatments. This research delves into the prevalence and characteristics of CPE in a tertiary hospital in Spain over the course of a three-year period, providing a comprehensive analysis of its typing and antimicrobial resistance determinants.

2. Results

2.1. Case Selection and Species Identification

From January 2021 to December 2023, a total of 38,145 *Enterobacteriaceae* were isolated in the clinical microbiology laboratory at HUMS. Approximately 97% of these (36,956 isolates), were obtained from clinical samples, with *Escherichia coli* (60.5%), *Klebsiella pneumoniae* (17.1%), *Proteus mirabilis* (6%), and *Enterobacter cloacae complex* (4.1%) being the most prevalent species. During this period, 112 CPE were identified, representing 0.4% of the total isolates. Besides, the distribution of both the total number of isolates and CPE was not uniform throughout the study period, as there was a consistent increase in the total number of enterobacteria and CPE. Specifically, the total number of enterobacteria increased by 18.1% in 2022 compared to 2021 and by 19.3% in 2023 compared to the previous year. However, the increase in CPE was greater than what could be explained solely by the increase in the total number of enterobacteria isolates, almost tripling in 2022 compared to 2021, representing 0.2% (p value <0.01) of the total isolates, and doubling again in 2023, representing 0.5% (p value <0.01) of total enterobacteria. Total isolations per species, year and presence of carbapenemase is presented in Table 1.

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Table 1. Distribution of Enterobacteriaceae isolation.

Species	2021	2021 carba	2022	2022 carba	2023	2023 carba	Total
E. coli	6629	0	7590	7	8870	14	23089
K. pneumoniae	1646	5	2102	14	2784	40	6532
P. mirabilis	649	0	741	0	891	1	2281
E. cloacae	424	3	542	2	579	3	1545
K. oxytoca	324	0	389	0	405	2	1118
Citrobacter spp	280	5	357	7	455	7	1092
S. marcescens	205	0	217	0	246	1	668
M. morganii	177	0	261	0	284	0	722
K. aerogenes	142	0	162	0	179	0	483
P. stuartii	42	0	60	1	70	2	172
Others	110	0	126	0	207	0	443
Total	**10628**	**13**	**12547**	**31**	**14970**	**70**	**38189**

Total isolation of Enterobacteriaceae by year and presence of carbapenemases. Carba: Isolations with carbapenemases.

The criteria for selecting CPE isolates were the first isolate per patient, year, species, and type of carbapenemase. Consequently, the 112 CPE isolates corresponded to 93 unique patients, of whom 74.1% were male. Patients' age ranged from 0.6 to 90.7 years, with a median age of 51.2 years. Epidemiological samples, mainly rectal and perianal swabs, accounted for 68% of the isolates, while the remaining 32% were from clinical samples. Among the clinical samples, approximately 50% were urine, 25% were from surgical wounds, and the remaining 25% were from abscesses, tracheal exudates, other wounds types, ascitic fluid, and blood. Most isolates were obtained from hospitalised patients, with 17% from patients admitted to an intensive care unit. Moreover, 66% of the isolates were from HUMS, and the remaining 34% were from other hospitals for which HUMS serves as a reference centre. 110 ff the 112 CPE, had reported antibiotic susceptibility testing, and 91 were available for sequencing (81%). For the remaining 21 strains, there was no viable sample to re-culture for sequencing.

K. pneumoniae was the most common CPE species, accounting for 51.8% of the isolates, followed by *E. coli* (18.8%), *Citrobacter spp* (16.1%), *Enterobacter cloacae* complex (7.1%), *Providencia stuartii* (2.7%), *Klebsiella oxytoca* (1.8%), *Proteus mirabilis* (1%), and *Serratia marcescens* (1%). This species distribution was comparable in the subset of CPE isolates that underwent WGS, consisting of *K. pneumoniae* (n=53), *E. coli*(n=21), *E. cloacae* (n=7), *Citrobacter spp* (n=7), *P. stuartii* (n=3), *K. oxytoca* (n=2) and *P. mirabilis* (n=1)

WGS yielded concordant species identification compared to Matrix-assisted laser desorption ionization time of flight (MALDI-TOF), while providing enhanced resolution to distinguish closely related species such as *K. oxytoca* and *K. michiganensis* or within complexes such as in *Citrobacter freundii* complex, in which two isolates were reclassified as *Citrobacter cronae* and *Citrobacter partucalensis*.

2.2. Antibiotic Susceptibility

The antibiotic susceptibility analysis showed high rates of resistance among CPE isolates. Resistance to cephalosporins such as ceftazidime and cefepime was observed in 77.5% and 84.3% of isolates, respectively. For carbapenems, resistance to ertapenem, imipenem, and meropenem reached 92.1%, 76.4%, and 68.5%, respectively. Approximately three-quarters of the isolates were resistant to the fluoroquinolones ciprofloxacin and levofloxacin. Aminoglycoside resistance ranged from 52.8% for gentamicin to 64% for amikacin and 75.3% for tobramycin. Resistance to the "last-resort" antibiotics fosfomycin, tigecycline, and colistin was 39.3%, 33.3%, and 10.6%, respectively. Additionally, the new generation antibiotics ceftolozane-tazobactam, ceftazidime-avibactam, and cefiderocol showed non-susceptibility rates of 77.5%, 41.6%, and 31.7%, respectively. These resistance patterns were analysed considering the intrinsic resistance profiles of the *Enterobacteriaceae* species evaluated, such as the presence of chromosomal AmpC in some of them. The fosfomycin results were limited to *E. coli* and *Klebsiella spp*., and the tigecycline cutoff was based on the *E. coli* interpretation. A detailed description of the antibiotic susceptibility pattern by carbapenemase type in each isolate is shown in Figure 1.

 doi:10.20944/preprints202412.0503.v1

Figure 1. Heatmap of antibiotic susceptibility pattern by isolate. Heatmap showing susceptibility profiles and carbapenemase of each isolate. Green: Susceptible, Red: Non-susceptible, Gray: Not Available, Yellow: Area of Technical Uncertainty. ID: Sample identification number CAZ: Ceftazidime, CFP: Cefepime, ERT: Ertapenem, IMI: Imipenem, MER: Meropenem, ATM: Aztreonam, CTO: Ceftolozane-Tazobactam, CZA: Ceftazidime-Avibactam, CFD: Cefiderocol, CIP: Ciprofloxacin, LEV: Levofloxacin, GEN: Gentamicin, TOB: Tobramycin, AMK: Amikacin, COL: Colistin, FOT: Fosfomycin, TIG: Tigecyclin.

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2.3. Resistome

The resistome annotation results encompassed all resistance mechanisms detected in the genomes. However, the analysis focused only on those mechanisms involved in direct modification of the antibiotic molecule or its target site of action, excluding resistance mechanisms related to cell membrane permeability, active efflux, and lipopolysaccharide profile.

In total, 101 carbapenemase-encoding genes were identified from the 91 genomes sequenced. The most common carbapenemase found was OXA-48-like, represented by 50 isolates with different alleles of the bla$_{oxa}$ gene i.e *bla*$_{OXA-48}$ (38 isolates), *bla*$_{OXA-181}$ (5 isolates), *bla*$_{OXA-244}$ (5 isolates), and *bla*$_{OXA-484}$ (2 isolates). This was followed by NDM, with two alleles present, *bla*$_{NDM-1}$ (15 isolates) and *bla*$_{NDM-5}$ (9 isolates). The third most common was the VIM-type carbapenemase, mainly *bla*$_{VIM-1}$ (13 isolates). The least frequent was the class A KPC carbapenemase, with a similar distribution between *bla*$_{KPC-2}$ (7 isolates) and *bla*$_{KPC-3}$ (6 isolates) alleles. Notably, ten strains were found to harbor dual carbapenemase genes, comprising *bla*$_{NDM-1}$ + *bla*$_{OXA-48}$ (7 isolates), *bla*$_{NDM-1}$ + *bla*$_{OXA-244}$ (2 isolates), and *bla*$_{NDM-1}$ + *bla*$_{KPC-2}$ (1 isolate).

Regarding other β-lactamases, extended-spectrum β-lactamase genes were detected in 47.3% of the isolates, with the CTX-M-type being the most common, including *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-9}$, and *bla*$_{CTX-M-55}$ alleles. Interestingly, the gene *bla*$_{VEB-6}$ was detected in *P. mirabilis.* No mutations at positions 238 or 179 in the *bla*$_{SHV}$ gene, conferring resistance to third-generation cephalosporins [15] were observed. Because their epidemiological importance, the presence of class C β-lactamase genes in the genomes of *E. coli* and *K. pneumoniae* was evaluated, excluding the *bla*$_{EC}$ gene due to its potential chromosomal nature in *E. coli.* Several alleles of the *bla*$_{CMY}$ gene were detected in seven *E. coli* strains, and *bla*$_{DHA-1}$ was found in one *K. pneumoniae* strain. Other β-lactamases detected in the isolates, mainly class A and D with lower hydrolytic profiles, included multiple alleles of *bla*$_{SHV}$, *bla*$_{TEM}$, and *bla*$_{OXA}$ genes. In particular, *bla*$_{LEN-16}$ was associated with *bla*$_{VIM-24}$ and *bla*$_{CTX-M-9}$ in one isolate of *Klebsiella variicola*. Only three strains exhibited a carbapenemase gene as the sole β-lactamase in their genome, two *Providencia stuartii bla*$_{NDM-5}$ and one *Citrobacter amalonaticus bla*$_{VIM-1}$. Table 2 shows carbapenemases types by species.

Table 2. Distribution of carbapenemases by species.

Carbapenemase/Species	*K. pneumoniae complex*	*E. coli*	*E. cloacae complex*	*Citrobacter spp*	*P. stuartii*	*K. oxytoca*	*P. mirabilis*	Total
KPC-2	2	1	0	3	0	0	0	6
KPC-3	4	0	2	0	0	0	0	6
NDM-1	4	0	0	0	0	0	1	5
NDM-5	0	6	0	0	3	0	0	9
VIM-1	3	3	2	3	0	2	0	13
VIM-24	1	0	0	0	0	0	0	1
OXA-48	24	3	3	1	0	0	0	31
OXA-181	4	1	0	0	0	0	0	5
OXA-244	1	2	0	0	0	0	0	3
OXA-484	0	2	0	0	0	0	0	2
NDM-1 + OXA-48	7	0	0	0	0	0	0	7
NDM-1 + OXA-244	0	2	0	0	0	0	0	2
NDM-1 + KPC-2	1	0	0	0	0	0	0	1
Total	51	20	7	7	3	2	1	91

Table 2 shows the distribution of carbapenemases according to the host microorganism. Total number of carbapenemases and Enterobacteriaceae ae showed in the last column and row, respectively.

Regarding fluoroquinolone resistance, 82.4% of the strains harboured genetic resistance determinants, including point mutations in the *gyrA*, *gyrB*, or *parC* genes, as well as the presence of the *qnr* gene, mainly multiple alleles of *qnrB*, and lower frequencies of *qnrA*, *qnrD*, and *qnrS*. The concurrent presence of at least one mutation in *gyrA* and *parC*, observed in 55 isolates, conferred phenotypic resistance in 100% of the cases. In contrast, the isolated presence of the *qnr* gene in 13 isolates resulted in variable levels of resistance and discrepant phenotypes, with some strains being ciprofloxacin-resistant but levofloxacin-sensitive. Additionally, six isolates had mutations in a single

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gene, either *gyrA* or *gyrB*, while 29 isolates had mutations in *gyrA*/*gyrB*, *parC*, and the simultaneous presence of *qnr*. The most common amino acid variant in GyrA was the substitution of serine for isoleucine at position 83, accounting for 43.9% of the cases, with less frequent changes to phenylalanine, leucine, and tyrosine at the same position, as well as other substitutions at position 87. Notably, the D87G substitution exhibited a fluoroquinolone-resistant phenotype, even in the absence of other resistance determinants. Substitutions in GyrB involved the change of glutamate to aspartate and serine to alanine at positions 463 and 466, respectively, the former being found only in the absence of *gyrA* mutations and conferring a resistant phenotype only when combined with the presence of the *qnr* gene. All substitutions in ParC corresponded to a change from serine to isoleucine at position 80, and no strains presented isolated mutations in the *parC* gene.

Genes encoding aminoglycoside-modifying enzymes were detected in approximately 90% of the isolates, with acetyltransferases being the most common type, followed by nucleotidyltransferases and phosphotransferases. More than one of these enzyme coding genes were found in two-thirds of the isolates, and 30% harboured three or more. The presence of the gene encoding AAC6'-Ib, an enzyme of epidemiological and clinical importance due to amikacin inactivation [16], was specifically investigated and detected in 56 isolates, 50% of which exhibited *in vitro* resistance to amikacin. The high diversity in the presence and combinations of genes of aminoglycoside-modifying enzymes made it challenging to correlate with specific *in vitro* susceptibility patterns to gentamicin, tobramycin, and amikacin, which were variable.

2.4. Typing

Multilocus sequence typing analysis revealed the presence of predominant sequence types among *K. pneumoniae* isolates, while the less common species exhibited greater genetic diversity. The most prevalent *K. pneumoniae* sequence type was ST147, followed by ST307 and ST395. Additionally, two isolates of the hypervirulent *K. pneumoniae* sequence type ST23 [17] were identified, which also harboured dual carbapenemase genes. In the case of *E. coli*, according to the Achtman MLST scheme [18], only two sequence types, ST2659 and ST167, were detected in two or more isolates, with four and two isolates, respectively. Interestingly, one *K. michiganensis* isolate showed a potential new sequence type, characterised by a G159T transversion in the *mdh* gene, representing a previously undescribed allele [19].

The cgMLST analysis of the *K. pneumoniae* isolates, using a more relaxed threshold of 15 different alleles to distinguish individual clusters given the temporal separation of the isolates [20–22], revealed a high degree of genetic diversity Figure 2. The analysis identified nine clusters containing at least two isolates, but only two of these clusters included four or more isolates. Notably, there was no clear clonal relationship between isolates expressing the same carbapenemase gene, with the exception of the *bla*$_{\text{OXA-181}}$ variant, where the four identified strains showed a close genetic relationship. Interestingly, four of the strains harboring the dual carbapenemase genes *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$ were grouped within the largest cluster, which was predominantly composed of the ST147 sequence type. In contrast, the *E. coli* analysis, using the same clustering threshold, identified only two clusters that directly corresponded to the observed sequence types Figure3. Importantly, these *E. coli* clusters exhibited a clear association between the isolates and the specific carbapenemase genes, with the main cluster carrying *bla*$_{\text{NDM-5}}$ and the secondary cluster carrying the dual carbapenemase genes *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-244}}$. Notably, two isolates harbouring *bla*$_{\text{OXA-484}}$ were separated only by 35 alleles in the cgMLST and one of them was catalogued as a possible new ST due to a mismatch in the *adk* gene that needs to be confirmed by resequencing, being the closest match ST1722.

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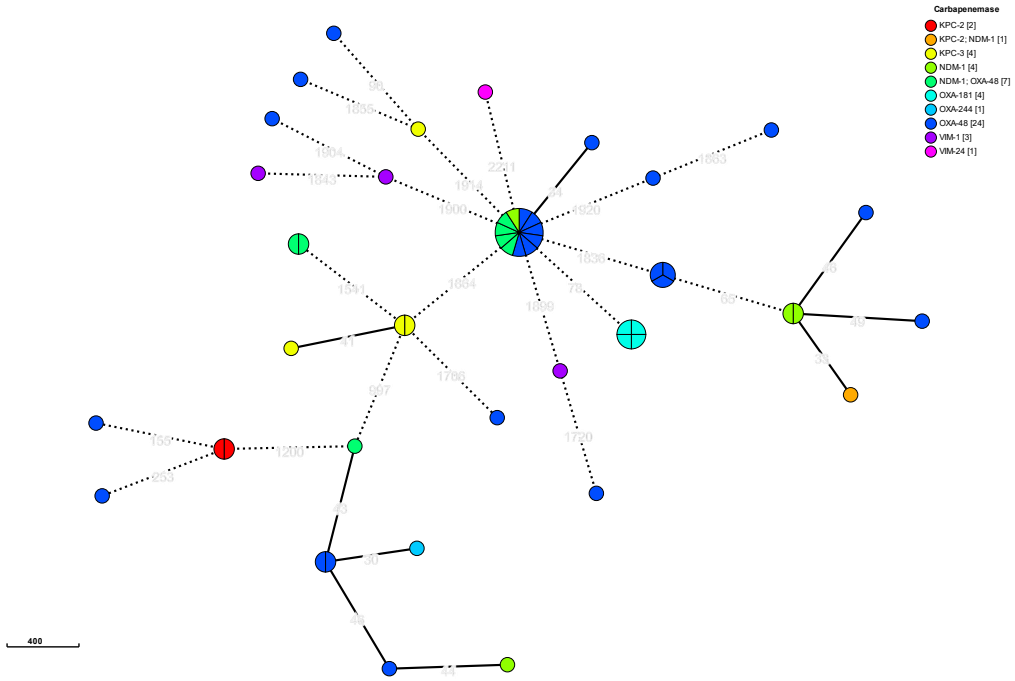

Figure 2. MST of the *K. pneumoniae* cgMLST. Minimum spanning tree reconstructed from the allele matrix of the cgMLST of K. pneumoniae species complex. Circles represent isolates, connected by solid lines with lengths proportional to the allelic distances between them. The allelic difference between each pair is indicated as an integer on the corresponding connecting line. Isolates with 15 or fewer allelic differences are grouped within the same circle, depicted as a pie chart, where the circle's size reflects the number of isolates it contains. Dashed lines indicate allelic differences greater than 50. The number in brackets in the legend indicates the absolute frequency of the observation.

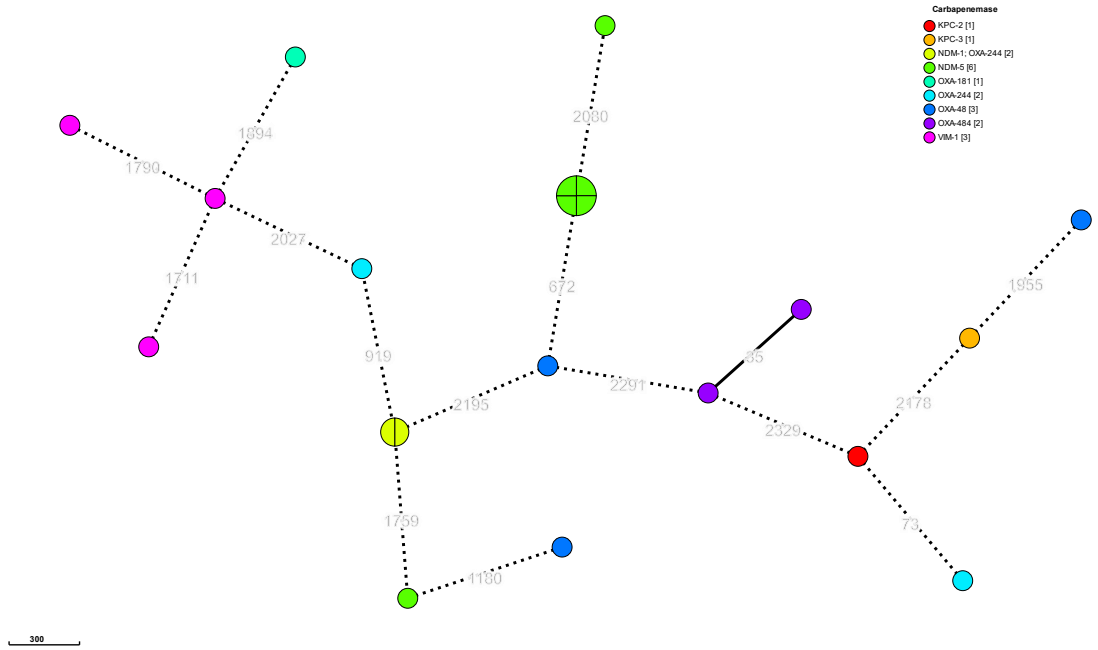

Figure 3. MST of the *E. coli* cgMLST. Minimum spanning tree reconstructed from the allele matrix of the cgMLST of E. coli. Follows the same schematics as in Figure 2.

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Phylogenetic analysis of the *K. pneumoniae* isolates revealed a distribution similar to the minimum spanning tree generated from cgMLST (Figures 4 and 5). However, a group of strains, in particular those belonging to sequence type ST512 and producing KPC-3 carbapenemase as well as a clade of heterogeneous ST395 isolates expressing diverse carbapenemase types, exhibited a large divergence in their ancestry, taking into account that the reference genome is ST11. Notably, two isolates (KP835819 and KP963668) belonging to the same high-risk ST (395) and harbouring a bla_{oxa-48} carbapenemase clustered together, although the patients had no epidemiological link to each other and the isolates were collected more than six months apart, highlighting the benefits of cgMLST for epidemiological tracking of CPE. Phylogenetic analysis of the *E. coli* isolates demonstrated a clear separation between strains producing VIM-1 and NDM-1 metallo-β-lactamases compared to the rest of the cohort. In addition, the formation of phylogenetically related clades was observed, taht were not apparent in the cgMLST analysis, particularly among strains harboring bla_{KPC} and *bla*$_{OXA-484}$ genes. The significance of these phylogenetic relationships would be determined by the epidemiological context surrounding the strains.

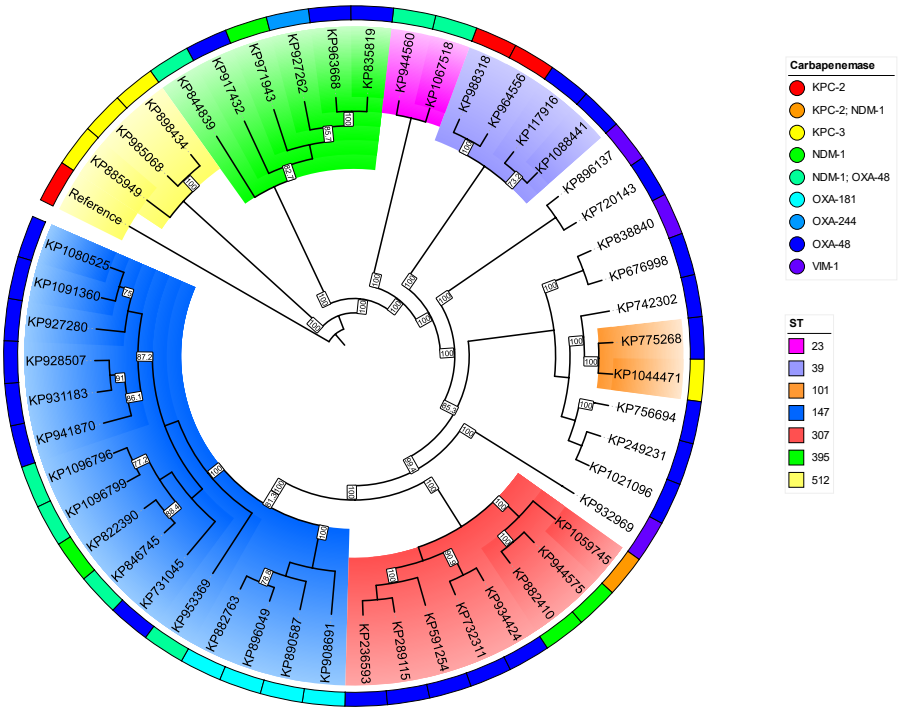

Figure 4. Phylogenetic analysis of carbapenem-producing *K. pneumoniae.* Phylogenetic analysis of K. pneumoniae sensu stricto. The cladogram depicts the relationships among isolates. Inner shading indicates the ST of each isolate. Isolates without shading represent unique STs. The outermost circle highlights the carbapenemase associated with each isolate. Bootstrap values exceeding 70 are displayed in white-background boxes on the respective branches.

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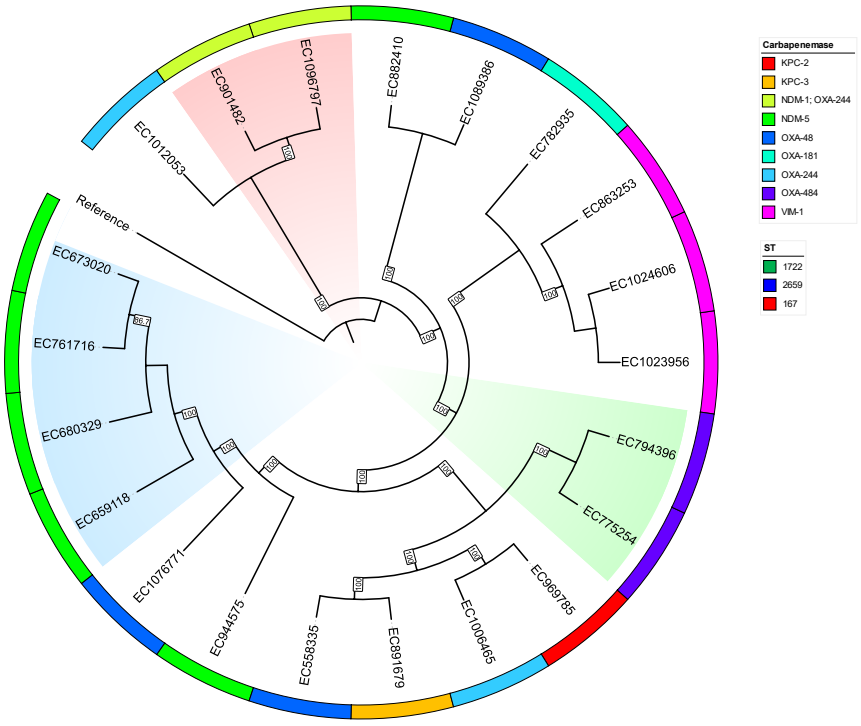

Figure 5. Phylogenetic analysis of carbapenem-producing *E. coli.* Phylogenetic analysis of E. coli. The cladogram depicts the relationships among isolates and follows the same schematics as in Figure 4.

2.5. Plasmids

The plasmid analysis focused on those associated with the presence of carbapenemase genes. Plasmids harbouring carbapenemase genes were identified in 93.4% of the isolates. For the *bla*$_{OXA-48}$ gene, the predominant replicon type was IncL, with an average size of 50kb, of which 50% were predicted to be conjugative, while the remaining 50% were non-mobilizable. In contrast, the *bla*$_{NDM-1}$ gene was primarily associated with IncF replicon type, which had an average size of 91.9kb, and 78.5% were conjugative, compared to 21.5% that were non-mobilizable. Overall, the most common plasmid replicon types were IncL and IncF, accounting for 60% of the samples. The average size of plasmids harbouring carbapenemase genes was 81Kb, ranging from 4.3Kb (*bla*$_{OXA-48}$) to 342.9Kb (*bla*$_{NDM-1}$), with 61.1% being conjugative, 4.7% mobilizable, and 34.1% non-mobilizable. Interestingly, more than one replicon was identified in 44.7% of the cases, and all plasmids larger than 80kb were predicted to be mobile.

Table 3 summarizes the molecular characterization of the CPE grouped by species.

Table 3. Genomic characterization summary of CPE.

CPE	Carbapenemases	ST	cgMLST cluster	BLEE	AmpC	*gyrA* mutation	*parC* mutation	Aminoglycosides modifing genes	Plasmid_replicons (carbapenemase)	Predicted_mobility	Mean plasmid size
K. pneumoniae	OXA-48	13,15,39,101, 147,307,346, 395,405,685 ,4872	Yes	CTX-M-15	0	S83I,S83F, D87A	S80I	Several	IncL/M,IncR	Variable	46443
	OXA-181	147	Yes	CTX-M-15	0	S83I	S80I	Several	rep_cluster_1195	Mainly non-mobilizable	40801
	OXA-244	395	No	CTX-M-15	0	S83I	S80I	Several	IncL/M	Conjugative	87763
	KPC-2	39	Yes	CTX-M-15	0	S83I,D87N	S80I	Several	IncFIB,IncFII,IncX3,ColRNAI_rep_cluster_1857	Mainly conjugative	94778
	KPC-3	101,512	Yes	0	0	S83I,S83Y, D87N	S80I	Several	IncFIB,IncFII,IncHI1B,IncR	Conjugative	63166
	NDM-1	147,307,395	Yes	CTX-M-15	0	S83I	S80I	Several	IncFIB,IncFII,IncHI1B	Conjugative	278576
	VIM-1	2,685,844,387	No	0	DHA-1	0	0	Several	IncL/M	Conjugative	71362

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	KPC-2 + NDM-1	307	No	0	0	S83I	S80I	aph(3')-VI only	IncFIB,IncHI1B,rep_cluster_1254	Conjugative	332589
	NDM-1 + OXA-48	23,147,395	Yes	CTX-M-15	0	S83I	S80I	Several	IncFIB,IncFII,IncHI1B	Variable	101820
E. coli	OXA-48	38,127,1598	No	CTX-M-15	0	S83L	0	Several	IncFIA,IncFII	non-mobilizable	21541
	OXA-181	410	No	0	CMY-4	S83L,D87N	S80I	Several	IncX3	non-mobilizable	27916
	OXA-244	44,13730	No	CTX-M-15	CMY-132	S83L,D87N	S80I	aadA5 only	ND	ND	ND
	OXA-484	1722	No	0	0	0	0	aadA2 only	rep_cluster_1195	non-mobilizable	14615
	KPC-2	131	No	0	CMY-132	S83L	0	Several	IncFIB,IncFII,rep_cluster_2183	Conjugative	84857
	KPC-3	135	No	0	0	0	0	APH(3') only	IncX3	Conjugative	58796
	NDM-5	46,405,2659	No	CTX-M-55	CMY-42	D87N, S83L	S80I	aadA2 only	IncFIA,IncFIC,IncFIB	Mainly conjugative	105572
	VIM-1	29,327,539	No	0	0	0	0	Several	IncL/M,IncL/M, IncI-gamma/K1	Variable	49209
	NDM-1 + OXA-244	167	Yes	CTX-M-15	0	D87N, S83L	S80I	AAC(3)-Iid only	IncC,rep_cluster_1254	Conjugative	210764
Citrobacter spp	OXA-48	225	ND	0	CYM-101	0	0	0	IncL/M	Conjugative	61961
	KPC-2	21259	ND	CTX-M-15,CTX-M-9	CMY-106,CYM-159	S83I	S80I	Several	IncP,IncU	Monilizable	29296
	VIM-1	488493563	ND	CTX-M-9	CMY-2,CMY-48	0	0	Several	IncL/M,IncY	Mainly conjugative	57471
Enterobacer spp	OXA-48	13,24	ND	CTX-M-15	ACT-1,MIR-5	S83I	0	0	IncL/M	Conjugative	64843
	KPC-3	51	ND	0	ACT-40	0	0	Several	IncN	Conjugative	50662
	VIM-1	108198	ND	CTX-M-9	ACT-55	0	0	Several	IncL/M,IncHI2A	Conjugative	152182
Klebsiella spp	VIM-1	59	ND	CTX-M-9	0	S463A	0	Several	IncL/M,IncR	Conjugative	174918
	VIM-24	4365	ND	CTX-M-9	0	S463A	0	Several	IncHI2A,rep_cluster_1088	Conjugative	289821
Providencia stuartii	NDM-5	11,23	ND	0	CMY-16	D87G	0	Several	IncC,rep_cluster_1254	Variable	78040
Proteus mirabilis	NDM-1	446	ND	VEB-6	0	S463A	0	Several	ND	ND	ND

In double carbapenemase carriers, the plasmid description refers to the one harbouring the metallobetalactamase gene. BLEE: Extended spectrum betalactamase.

3. Discussion

From 2021 onward, a consistent and progressive annual increase of approximately 20% in the total number of Enterobacteriaceae isolates was observed in our hospital. This upward trend appeared to be strongly correlated with an increase in the volume of laboratory samples, particularly those sourced from primary care settings. This rise followed the reactivation of clinical services after the gradual de-escalation of COVID-19 restrictions. Notably, this surge in isolates was not observed in epidemiological samples collected between 2021 and 2022. During this period, the majority of epidemiological samples were collected from intensive care units that remained operational despite the pandemic-related restrictions, potentially limiting the increase. However, the significant escalation of Enterobacteriaceae isolates observed in 2023 may probably be attributed to the implementation and strengthening of surveillance programmes focused on multidrug-resistant (MDR) organisms in the community [23]. These programmes, introduced in 2022, played a crucial role in enhancing the detection and surveillance of MDR pathogens, thereby contributing to the increased identification of Enterobacteriaceae isolates in the following year.

In addition to the overall increase in Enterobacteriaceae isolates, the rise in CPE isolates was particularly pronounced, even after adjusting for the general increase in reported isolates over the same period. This significant increase highlight a notable shift in the local epidemiology of CPE, which may be attributed to several factors, including the introduction of strains from high-prevalence regions and/or the enhanced surveillance efforts focused on multidrug-resistant (MDR) organisms within hospital settings [24–26]. The species distribution of CPE isolates also differed significantly from global patterns, with *K. pneumoniae* being the predominant species, followed by *E. coli*, *Citrobacter spp.*, and *E. cloacae* complex. This deviation from global trends has been documented in other studies and may reflect species-specific traits that enhance the ability of these organisms to adapt to hospital environments [27,28]. In addition, the prevalence of carbapenemase production in these species may be facilitated by horizontal gene transfer mechanisms, that promote the spread of resistance traits, further contributing to the evolving local epidemiology of CPE [29–31].

 doi:10.20944/preprints202412.0503.v1

Although the majority of CPE isolates were obtained from epidemiological samples, it is noteworthy that several patients developed infections requiring antibiotic treatment for the same CPE strains they initially carried [32,33]. However, this clinical progression is not fully captured in the data, as only the first isolate received in the laboratory was included in the analysis. This methodological limitation means that subsequent infections or isolates from the same patients, which could reflect the development of clinically significant infections or changes in resistance profiles, were not accounted for. Consequently, the data may not fully reflect the dynamic nature of CPE colonization and infection in the patient population over time.

Consistent with findings from previous studies [34,35], a high level of resistance to the major classes of antibiotics commonly used in clinical practice was observed, particularly against third- and fourth-generation cephalosporins [36,37], fluoroquinolones [38], and aminoglycosides [39]. This pattern of multidrug resistance is likely attributed to several interrelated mechanisms, including the hydrolytic activity of carbapenemases that, target and degrade other beta-lactams, the co-occurrence of genes encoding extended-spectrum beta-lactamases (ESBLs), and aminoglycoside-modifying enzymes on either the same or separate plasmids. Additionally, mutations in the *gyrA* and *parC* genes, which are selected by prior fluoroquinolones exposure, contribute to resistance [40]. Interestingly, the relatively low rate of meropenem resistance observed in our CPE isolates may be explained by the high prevalence of class D carbapenemases. These enzymes show variable sensitivity to meropenem, which, although effective against certain carbapenemases, remains an unsuitable therapeutic option due to its reduced efficacy when facing these enzymes, despite showing low minimum inhibitory concentrations (MICs) [41]. Resistance to new-generation cephalosporins was also notable, with a particularly high rate of resistance to ceftolozane-tazobactam. This resistance is probably due to the limited activity of ceftolozane-tazobactam in isolates expressing carbapenemases, despite its potent anti-Pseudomonas activity [42]. In contrast, resistance to ceftazidime-avibactam was found to be consistent with the prevalence of metallo-beta-lactamases, as avibactam is effective against class A and D carbapenemases but lacks activity against class B enzymes [43]. In contrast to the findings reported by Lasarte-Monterrubio et al. in other isolates from Spanish hospitals, the high rates of cefiderocol-resistant strains observed in this study is remarkable. This is particularly noteworthy given that cefiderocol, a recent addition to the antibacterial armamentarium, is especially intended to treat multidrug-resistant strains infections, including CPE. Mutations in genes that regulate iron transport and the expres-sion of the carbapenemase VIM-1 have been proposed as factors contributing to cefidero-col resistance [44]. However, due to limited knowledge of resistance determinants and the need for strain-specific analysis, these factors were not included in the resistome analysis, though further investigation in future studies would be very valuable.

Despite fosfomycin and tigecycline exhibited lower resistance rates compared to other antibiotics, more than one-third of isolates were non-susceptible to these agents. These antibiotics, along with colistimethate, have limited therapeutic efficacy and a higher risk of adverse effects [45], underscoring the need for tailored treatment strategies when managing CPE infections. Given these challenges, antibiotic susceptibility testing and carbapenemase characterization remain essential to guide appropriate and effective therapeutic interventions in the management of CPE infections.

The high prevalence of *K. pneumoniae* producing OXA-48 carbapenemase observed in this study is consistent with both local and national epidemiological data, as reported by the Spanish Society of Infectious Diseases and Clinical Microbiology [46]. However, an emerging concern is the increasing diversity of carbapenemase types over time, including several alleles of $bla_{OXA\text{-}48}$ as well as class A and B carbapenemases. This trend highlights the evolving complexity of resistance mechanisms within the local epidemiology of multidrug-resistant organisms. Notably, no single carbapenemase type was found to be exclusive to a particular species, suggesting a broader dissemination of these resistance traits across different bacterial species. This widespread distribution of carbapenemase-producing strains further emphasises the need for active surveillance and comprehensive strategies to mitigate the spread of resistance.

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The identification of key sequence types (STs) in *Klebsiella pneumoniae*, particularly high-risk clones such as ST147 and ST307, is consistent with previous reports of global outbreaks and the spread of carbapenemase-producing strains [47]. These STs are well-documented in the spread of carbapenemase resistance, highlighting the global nature of this problem [48,49]. Notably, two strains belonging to the hypervirulent ST23 clone were found to exhibit dual carbapenemase production, specifically *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$, located on separate plasmids. This is the first reported instance of such an association in ST23, demonstrating the evolving complexity of resistance mechanisms and the need for enhanced genomic surveillance programmes in healthcare settings. The emergence of multidrug-resistant hypervirulent strains is of particular concern, as these isolates are associated with increased morbidity and mortality, representing a significant public health threat [50,51].

Interestingly, in contrast to the findings of other epidemiological surveillance studies on carbapenem-resistant *Escherichia coli*, which commonly identify ST131, ST38, and ST405 as predominant clones [52,53], our study revealed a different pattern. The isolates in this study exhibited marked heterogeneity in sequence types, with ST2659 being the most prevalent. Recently described in Algeria and Nepal [54,55], ST2659 is associated with *blaNDM* alleles and belongs to clonal complex 38, a group that has been linked to the global dissemination of carbapenemase-producing bacteria. The presence of ST2659 in this context further emphasises the dynamic and evolving nature of carbapenemase-producing bacteria, and the global spread of resistance determinants across different bacterial species and geographical regions.

Our study underlines the widespread presence of plasmids harboring carbapenemase genes, with 93.4% of isolates carrying such plasmids. The absence of plasmid identification in the remaining isolates probably reflects the limitations of only short-read sequencing for draft assembly. These plasmids may have been split into multiple fragments in smaller contigs during assembly or may be present in low copy numbers within the isolates. It is plausible that the combination of current data with long-read sequencing could facilitate the accurate characterisation of the mobile elements carrying the carbapenemase genes in these isolates. The predominant replicon types for blaOXA-48 and blaNDM-1 were IncL and IncF, respectively, with different mobilisation characteristics. Notably, 50% of blaOXA-48 associated plasmids were non-mobilizable, whereas a significant 78.5% of blaNDM-1 plasmids were conjugative. The identification of multiple replicons in nearly half of the cases highlights the complexity of carbapenemase gene transfer. The high mobility of plasmids larger than 80kb suggests the potential for rapid horizontal gene transfer, which may contribute to the spread of resistance in clinical settings.

4. Materials and Methods

4.1. Strains Included in the Study and Criteria of Selection of CPE

Data on the number, species, and antibiotic susceptibility profiles of Enterobacteriaceae isolates obtained at HUMS from January 2021 to December 2023 were extracted from the laboratory's electronic records (HORUS).

Antibiotic susceptibility testing was performed by broth microdilution method, using the MicroScan™ WalkAway semi-automated system (Beckman Coulter) with the exception of cefiderocol testing that was performed by Kirby-Bauer method. Results were interpreted according to the The European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [56]. Inconclusive results were confirmed by Kirby-Bauer or MIC test strip methods (Liofilchem®).

All CPE isolates were recovered from human clinical or epidemiological samples submitted to the HUMS Microbiology Laboratory as part of routine diagnostic procedures.

Screening for carbapenemase production was performed in all strains that presented a MIC of meropenem and or ertapenem >=0.125 mg/L [57]. CPE in clinical samples were identified either by immunochromatographic assays NG-Test® CARBA 5 (NG-Biotech Laboratories) or genotypically by isothermal amplification techniques (Eazyplex® AmplexDiagnostics GmbH) or FilmArray® (BioFire Diagnostics), depending on the specific case and in accordance with laboratory protocols. All strains

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with a positive molecular result for carbapenemase detection were considered CPE, regardless of their minimum inhibitory concentration to carbapenems.

Epidemiological samples were initially screened for CPE using chromogenic selective media *Brilliance*™ CRE (Oxoid Limited), followed by a lateral flow assay NG-Test® CARBA 5 (NG-Biotech Laboratories) or real-time polymerase chain reaction (Xpert® Carba-R, Cepheid) confirmation of positive isolates.

Species-level identification was conducted using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) (Bruker Daltronik GmbH) from solid medium cultures, following manufacturer instructions.

4.2. WGS of CPE Isolates

All identified CPE isolates were candidate for WGS. To increase the available biomass, a subculture was prepared on Columbia blood. Genomic DNA was then extracted from the subculture using a magnetic capture-based method with a MagCore® system (RBC Bioscience), following the manufacturer's protocol, yielding 60 µL of eluate. The extracted genomic DNA was used to construct sequencing libraries using the Nextera XT™ Illumina® kit. Sample quantity and quality was verified at each step of the library preparation process using Qubit™ (Thermo Fisher Scientific, Inc) fluorometric quantification and Bioanalyzer™ (Agilent Technologies, Inc) analysis. Sequencing was performed on an Illumina® MiSeq™ instrument using MiSeq V2 300 reagent cartridges, employing a 150-base paired-end protocol and an expected average sequencing depth of greater than 30x.

De novo assembly was performed using Unicycler v0.5.0 [58], and the structural and functional quality of the assemblies was evaluated using QUAST v5.2.0 [59] and BUSCO v5.6.1 [60], respectively. Samples were rejected based on the following quality criteria: fewer than 800,000 reads with a quality score below 28 on the Phred scale, more than 10% Ns, N50 less than 30,000, or a genome size outside the range of 5.5 Mb ± 1.5 Mb. The presence of contaminants in the raw reads and assembled genomes was assessed using Mash v2.3 [61] and GUNC v1.0 [62], respectively. Finally, the assembly graphs were manually inspected using Bandage v0.8.1 [63]. Draft assemblies are under BioProject accession: PRJNA1190923

Species-level identification was verified using GAMBIT v1.0.1 [64] and the online PubMLST species identification service (https://pubmlst.org/species-id). Multi-locus sequence typing (MLST) was performed using mlst v2.23.0 [65], except for *K. pneumoniae*, which was analyzed separately. MLST and annotation of antimicrobial resistance and virulence genes for *Klebsiella* sp was performed with kleborate v2.4.1 [66]. Structural annotation was performed with prokka v1.14.6 [67], functional annotation with sma3s v2 [68] (Uniref90 database), and resistance mechanisms were annotated using rgi v6.0.3 [69] (CARD v3.2.9 database) and abricate v1.0.1 [70,71] (ResFinder database). Resistance mechanisms with coverage >80% and identity >95% were considered.

The phylogenetic analysis was based on the alignment of single nucleotide variants (SNVs) identified between the studied samples and the NCBI reference genome assemblies GCF_000240185.1 and GCF_000005845.2 for *K. pneumoniae* and for *E. coli*, respectively. Mapping, variant calling, and coreSNV calculation were performed using snippy v4.6.0 [72]. Recombination events were managed using the gubbins software v2.3 [73]. Phylogenetic inference and reconstruction were conducted using a maximum likelihood approach, with 1000 ultrafast bootstrap replicates and 1000 approximate likelihood-ratio tests. The substitution models employed were TVM+F+ASC+R3 for *E. coli* and TVM+F+ASC+G4 for *K. pneumoniae*, implemented in iqtree v2.2.6 [74–76].

Multilocus sequence typing of the core genome (gcMLST) was carried out for *E. coli* and *K. pneumoniae* using chewBBACA v3.3.1 [77] and the core genome schemes available from the website https://cgmlst.org/ncs website. Training data files were generated using prodigal v2.6.3 [78] with the same reference sequences as those employed in the phylogenetic analysis. The results of the cgMLST analysis were then visualised by constructing a minimum spanning tree, created using the MSTreeV2 algorithm within the grapetree v2.2 software [79].

The plasmidome was annotated by homologous sequence comparisons using the abricate v1.0.1 [70,80] software (PlasmidFinder database). Reconstruction and assembly of the plasmidome were

 doi:10.20944/preprints202412.0503.v1

conducted using MOB-suite v3.1.8 [81] and plasmidSPAdes v3.15.5 [82]. Further plasmidome annotation was performed using bakta v1.8.2 [83] (DB light v5.0 database) and rgi 6.0.3 [69], with visualization in the proksee software [84].

Nucleotide identity analyses were carried out employing fastANI v1.34 [85].

Inferential statistical analysis (Z-test to evaluate the difference between proportions) was performed using the prop_test function of the rstatix package in R v4.4.2.

5. Conclusions

The escalating prevalence of CPE in healthcare settings is an immediate and pressing public health challenge. This trend is particularly alarming given the limited therapeutic options available to treat CPE infections, which are often resistant to second- and third-line therapies. In light of our findings, the increase in CPE prevalence, from 0.2% to 0.5% of total Enterobacteriaceae isolates between 2021 and 2023, signals the need for ongoing and systematic surveillance to understand and address the dynamics of resistance. In this context, genomic characterisation, in particular by WGS, is essential. This work also highlights the valuable information that WGS of CPE in a tertiary hospital setting could provide, including an in-depth understanding of the genetic determinants of resistance, high-resolution species identification and delineation of strain-level relationships, which in turn can be used by clinicians and public health authorities to guide therapy and take action to prevent or mitigate potential outbreaks.

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Informed Consent Statement: Exemption of informed consent for the collection of retrospective data was accepted by the local institutional ethics committee "Comité de Ética de la Investigación de la Comunidad Autónoma de Aragón (CEICA)" protocol code PI24/155 and approval date 17/04/2024 in record Nº 08/2024.

Data Availability Statement: Data were submitted to GenBank on 11/26/2024 under ID SUB14891856. Approval and assignment of accession numbers are pending. If necessary, a complete list of GenBank and/or BioSample accessions can be provided in a supplementary table. BioProject accession: PRJNA1190923

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